*ENERGY-LINKED REACTIONS IN PHOTOSYNTHETIC BACTERIA, V. RELATION OF THE LIGHT-INDUCED PROTON UPTAKE TO PHOTOPHOSPHORYLATION IN R. RUBRUM CHROMATOPHORES**

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Abstract.—The kinetics of photophosphorylation have been studied in *Rhodospirillum rubrum* chromatophores. No evidence for a time or intensity lag in photophosphorylation was found. An intensity lag could be induced with the uncoupling agent, *m*-chlorocarbonyl cyanide phenylhydrazone or, in some cases, by aging of the chromatophores.

In chloroplasts, the occurrence of a time and intensity lag in phosphorylation has been correlated with the formation of a proton gradient and quoted as evidence that a proton gradient is a prerequisite of phosphorylation. The significance of the absence of a time and intensity lag in chromatophores is discussed in this context.

Introduction.—In a recent publication, Thore *et al.*[1] reported that the combination of two antibiotics, valinomycin and nigericin, inhibited photophosphorylation in chromatophores of *Rhodospirillum rubrum*, whereas either alone had little effect. In mitochrondria, valinomycin induces an energy-dependent K^+ uptake which is accompanied by a nearly stoichiometric efflux of H^+.[2] In chromatophores, valinomycin stimulates light-induced proton uptake which is dependent on K^+.[1, 3-4] In contrast, nigericin promotes a passive $K^+ \leftrightarrow H^+$ exchange and thus dissipates any K^+ or H^+ gradient formed under the influence of valinomycin.[4-7] These observations, coupled with others on the effect of these antibiotics on the kinetics of proton movement in chromatophores, led Thore *et al.*[1] to postulate that this combination of antibiotics stimulated a cyclic energy-requiring ion transport that competed with ATP formation for a high-energy intermediate ($I \sim X$) formed by light-induced electron transport.

Jackson *et al.*[4] reported similar results but interpreted them quite differently. They postulated that the combination of valinomycin and nigericin dissipated the pH gradient and membrane potential that is required for phosphorylation according to Mitchell's chemiosmotic hypothesis of the mechanism of ATP formation.[8] Currently available information does not resolve these two opposing views, although the results reported by Chance *et al.*[9] and Nishimura *et al.*[10] are more compatible with the former postulate.

Using chloroplasts, Schwartz[11] and Dilley[12] have reported experiments which apparently demonstrate that a critical level of proton accumulation must occur before ATP formation can take place. This was demonstrated in two ways. With very low light intensities a small amount of H^+ uptake occurred, but no ATP was formed even with prolonged illumination periods. The same relationship between the extent of H^+ uptake and ATP formation was found using nigericin[5] to control the extent of proton accumulation.

This apparent requirement for a threshold level of proton uptake before ATP synthesis begins in chloroplasts suggests that a time lag should be observed for photophosphorylation. Such a lag has been reported.[13, 14] This threshold requirement also suggests that a lag should be observed when the initial rate is plotted against the light intensity. Such a lag has also been reported.[12, 14–16]

Since these results apparently support the concept that a proton gradient is a prerequisite for photophosphorylation in chloroplasts, similar experiments were performed with chromatophores of *R. rubrum*. In the experiments reported in this paper, we found no evidence for either a time or intensity lag in photophosphorylation in this microorganism.

Methods.—R. rubrum, S1, was grown and, chromatophores were prepared as described[17] except that some preparations which were used for measuring pH changes were washed and suspended in 50 or 100 mM KCl. Changes in pH were measured aerobically in a stirred, water-jacketed, 3-ml reaction vessel at 28° with a Radiometer, model 22 pH meter with miniature pH electrodes. The sensitivity of the recordings was determined by the addition of known amounts of HCl. Red-light illumination was obtained with a Unitron LKR illuminator and a Corning 2403 red filter. The intensity was varied with calibrated Bausch and Lomb neutral density filters and optical density screens, and was measured with a Kettering YSI Radiometer. Reactions for the assay of ATP formation were terminated by the addition of trichloroacetic acid to 5%. AT^{32}P formation was assayed as previously described.[18] Valinomycin and nigericin were gifts from Dr. J. M. McGuire of the Lilly Research Laboratories.

Results and Discussion.—Time course of photophosphorylation: The traces (*A* and *B*) reported in Figure 1 indicate the time course of the light-induced pH change observed in a phosphorylating reaction mixture with *R. rubrum* chromatophores. The kinetics observed in curve *A* can be interpreted as an initial rapid pH rise due to a reversible light-induced proton uptake that has a half-time of 3–5 sec and a concomitant pH change associated with ATP formation.[19] Curve *B* represents the same system but with 0.43 μM nigericin; as previously demonstrated in this laboratory,[20] nigericin inhibited the initial rapid H^+-uptake but had little effect on photophosphorylation. If the formation of a proton gradient is a prerequisite for phosphorylation, a lag period in ATP formation should be evident while this gradient is being formed. From the original traces which are reproduced in Figure 1, we could detect a lag period of 0.5 sec or greater, if one existed. It is clear that no lag period was apparent even though this experiment

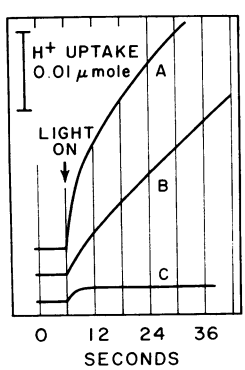

FIG. 1.—Light-induced pH changes. The reaction mixture contained 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM P_i, 0.02 mM succinate, and 96 μg Bchl in 3 ml at pH 7.4. A light intensity of 1.4×10^4 ergs cm^{-2} sec^{-1} was used. Curve *A*, 0.33 mM ADP; curve *B*, 0.33 mM ADP plus 0.43 μM nigericin, curve *C*, *N*-2-hydroxypiperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer equivalent in buffering capacity to ADP plus 0.43 μM nigericin. The rate of ATP formation in curve *A* was 30 μmoles/mg Bchl/hr which was 18% of the maximal rate at higher intensities.

was performed with a low light intensity (1.4×10^4 ergs cm^{-2} sec^{-1}) that would be expected to enhance a lag period if it were present. However, as is illustrated in curve *C* of Figure 1, nigericin did not completely eliminate the initial H^+-uptake, and in separate experiments we found that nigericin inhibited maximally the nonphosphorylating pH change by about 90 per cent. Thus, although these experiments do not exclude the possibility that the initial pH change observed in curve *B* is due to the nonphosphorylating pH change, it is unlikely that there is any lag in phosphorylation since the initial rate of the pH change observed in curve *B* is greater than the initial rate for curve *C*. This indicates that the initial rate of proton uptake observed in curve *B* is due to both phosphorylation and the nonphosphorylating pH change.

To ascertain further that there was no time lag in phosphorylation, we measured the kinetics of phosphorylation using the incorporation of $^{32}P_i$ into ATP. The time course of photophosphorylation under four different conditions is illustrated in Figure 2. In curve *A*, ascorbate was used to poise the system at the optimal oxidation-reduction potential. In curve *B*, both the light intensity (2.4×10^4 ergs cm^{-2} sec^{-1}) and a pH (6.4) used were suboptimal. In curve *C*, *N*-methylphenazinium methyl sulfate was used as a cofactor but with no reducing agent present to poise the system. No lag was apparent in any of these experiments. Furthermore, even when valinomycin was added (curve *A* + Val), there was again no lag even though this antibiotic stimulates the initial rate of light-induced proton uptake by severalfold at this concentration.[1, 3, 4] (The inhibition due to valinomycin is not related to its ion transport-inducing properties. It apparently inhibits electron transport at concentrations greater than 10^{-7} *M* (unpublished observations).)

These results are somewhat surprising whether we postulate (I) a requirement of a proton gradient for ATP formation or (II) that ion transport is an energy-linked reaction competitive with ATP formation. In the first case, a lag would be expected plus or minus valinomycin. In the second case, in a representative experiment valinomycin at 4.5×10^{-7} *M* stimulated the proton pump by about 4.5-fold at the light intensity (saturating) and pH used in the experiment represented in curve *A*. A typical initial rate of the light-induced pH change in KCl is 234 μmoles H^+/mg bacteriochlorophyll (Bchl) per hour. Thus, the introduction of 4.5×10^{-7} *M* valinomycin stimulated the rate to 1050 μmoles H^+/mg

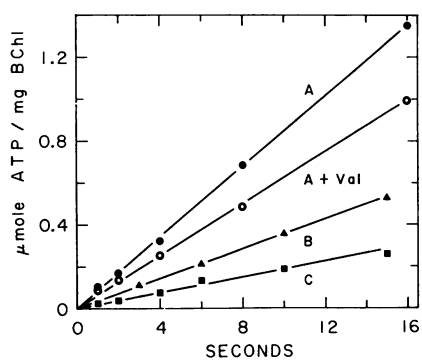

FIG. 2.—Time course of photophosphorylation. The reaction mixture contained 50 mM HEPES, 100 mM KCl, 1 mM $MgCl_2$, 3.3 mM $^{32}P_i$, 0.83 mM ADP, and approximately 62 μg Bchl in addition to those described below. (*A*) pH 7.4, 2 mM sodium ascorbate, and 0.45 μM valinomycin where indicated; light intensity was 1.6×10^5 ergs cm^{-2} sec^{-1}. (*B*) pH 6.4, 1.67 mM succinate, and the light intensity was 2.4×10^4 erg cm^{-2} sec^{-1} (55% maximal). (*C*) 0.02 mM *N*-methylphenazinium methyl sulfate, pH 7.4, and the light intensity was 2×10^5 ergs cm^{-2} sec^{-1}. The rate in curve *C* was low, since no reducing agent was included to poise the system.

Bchl per hour. This increased energy consumption should have been reflected in an inhibition of ATP formation during the time period of formation of the proton gradient. Thus, if we assume that one high-energy bond is required to transport four H^+,[2, 21] then 205 μmoles equivalent of ATP would be required to support this increased H^+ transport that was due to valinomycin. The initial rate of ATP formation of curve *A* in Figure 1 was 304 μmoles ATP/mg Bchl per hour. Thus, the rate should have been inhibited by 67 per cent if ion transport is competing with ATP formation for high-energy bonds formed by light-induced electron transport. Even if the stoichiometry of H^+ transported to high-energy bonds consumed is relatively high (and this is an unknown entity in this system), some inhibition of ATP formation should have been observed during the time period when the proton gradient was formed.

The lack of competition of ion transport with ATP formation is reminiscent of our results with the energy-linked transhydrogenase in *R. rubrum*.[22] We could not demonstrate any inhibition of ATP formation by transhydrogenation in this organism which indicated a lack of competition between these reactions. The same observation applied to the energy-linked NAD^+ reduction (unpublished observations). Conversely, ATP formation somewhat inhibited both the energy-linked transhydrogenase,[22] NAD^+ reduction,[23] and H^+ uptake as measured by bromothymol blue.[10] Although not entirely satisfactory, our interpretation is that light-induced electron transport has the capacity to produce more high-energy bonds than can be utilized by the phosphorylation of ADP. Thus ion transport, transhydrogenation, and NAD^+ reduction probably utilize energy that otherwise would be dissipated and are not competitive with ATP formation. On the other hand, ATP formation competes to a limited extent with the other energy-consuming reactions.

Effect of light intensity of the initial rate of the pH change and photophosphorylation: If there is a threshold proton gradient or membrane potential required for photophosphorylation in *R. rubrum*, a lag should be apparent at low intensities of illumination when the rate of ATP formation is plotted against the light intensity. In Figure 3 the light intensity curves for three separate experiments, run at different pH values and ADP and P_i concentrations, are compared with the effect on the proton pump under the same conditions. The curves all apparently extrapolate to the origin, indicating that no measurable lag can be seen in any of these experiments.

Thus, from these experiments we conclude that ATP formation in *R. rubrum* chromatophores does not require the development of a measurable membrane potential or pH gradient. These experiments do not exclude the possibility that chromatophores have a resting potential that is almost sufficient for ATP formation.

Induction of an intensity lag in photophosphorylation: In some aged chromatophore preparations in which the rate of phosphorylation was severely depressed, we observe a slightly *s*-shaped curve. This induction of a lag by aging was not reproducible consistently, but we found that an uncoupler such as *m*-chlorocarbonyl cyanide phenylhydrazone (*m*-Cl-CCP) would induce a marked intensity lag in ATP formation. This effect is illustrated in Figure 4. Thus, we

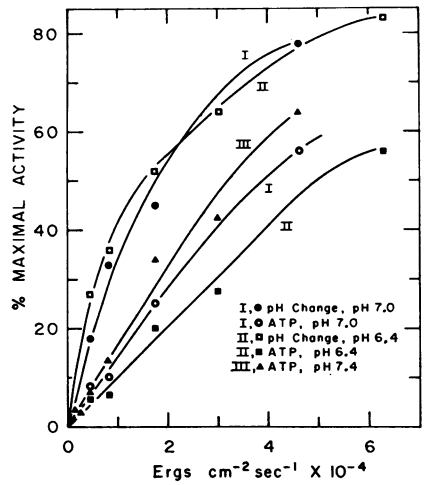

FIG. 3.—Effect of light intensity on ATP formation and the nonphosphorylating pH change. The reaction mixture for curve *I* contained 50 mM KCl, 1 mM $MgCl_2$, 0.02 mM succinate, and 68 μg Bchl; 0.33 mM $^{32}P_i$ and 0.17 mM ADP were included for the ATP curve. The maximal rates of ATP formation and H^+ uptake were 324 and 233 μmoles/mg Bchl/hr, respectively. The mixture for the pH change of curve II contained 50 mM KCl, 0.02 mM succinate, and 97.5 μg Bchl. The mixture for ATP formation for curves *II* and *III* contained 50 mM HEPES, 50 mM KCl, 1.67 mM succinate, 1 mM $MgCl_2$, 0.83 mM ADP, and 3.3 mM $^{32}P_i$. The rates of ATP formation and H^+ uptake for curves *II* were 223 and 239 μmoles/mg Bchl/hr, respectively. The reactions were illuminated for 1 min.

can speculate that any condition which emulates an uncoupling effect would induce a lag. Such conditions could be (*a*) aging which releases fatty acids that are uncouplers; (*b*) preparation conditions which cause membranes to become more permeable to ions, thus increasing the capacity for an energy-linked cyclic ion transport which would compete with ATP formation; or (*c*) aging or other conditions which induce ATPase activity.

Conclusions.—The kinetics of photophosphorylation and the light intensity-phosphorylation relationship reported in this paper do not support the postulate that a proton gradient, as measured by a pH change of the external medium with *R. rubrum* chromatophores, is required for photophosphorylation. Instead, we believe that ion transport represents a reaction that utilizes a high-energy intermediate ($I \sim X$) in the pathway of ATP formation as is illustrated in Figure 5. In this scheme, which represents the well-known chemical intermediate theory of

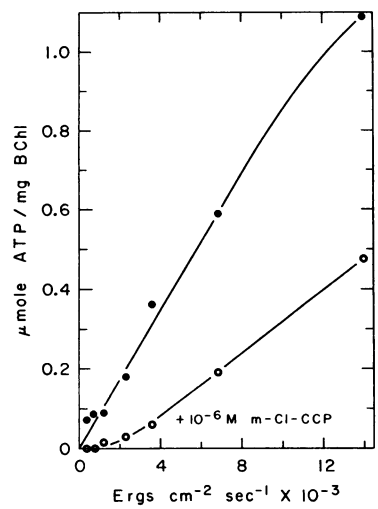

FIG. 4.—Induction of an intensity lag with *m*-chlorocarbonyl cyanide phenylhydrazone. The reaction mixture was as described for Fig. 2 and contained 0.02 mM *N*-methylphenazinium methyl sulfate, 70 μg Bchl, pH 7.4, and 10^{-6} *M* *m*-chlorocarbonyl cyanide phenylhydrazone. Narrow bandwidth illumination, centered around 870 mμ (Baird-Atomic interference filter), was used for this experiment.

phosphorylation, the intermediate ($I \sim X$) formed as a result of electron transport can be utilized to drive ion transport (reaction 1). In *R. rubrum*, it is not known whether cations (C^+) or protons (H^+) are the actively transported molecules.

In chloroplasts, the evidence which supports the concept that a proton gradient is the driving force for ATP formation is more convincing. However, by making two postulates, we believe the chloroplast experiments can also be explained by the above scheme: (Postulate I) The affinity of the proton pump (reaction 1) is greater than the affinity of ADP and P_i (reaction 2) for $I \sim X$; and (Postulate II) reaction 1 leading to the formation of a proton gradient is reversible, and thus a pH gradient can be used to synthesize ATP.

If postulate I is correct, a lag in the time-rate curve corresponding to the time of formation of the proton gradient would be expected since the initial rate of proton uptake is fast and initially consumes the $I \sim X$. As the proton pump approaches the steady-state, phosphorylation could begin to compete more effectively for $I \sim X$. This postulate can also explain the uncoupling effect of nigericin in chloroplasts. Nigericin induces a passive $K^+ \leftrightarrow H^+$ exchange which acts to dissipate the light-induced uptake of H^+. Thus, the steady-state rate of

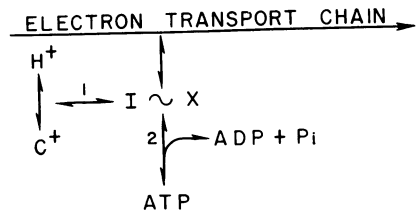

FIG. 5.—Postulated scheme of energy utilization.

H^+ uptake in the presence of nigericin is very fast and competes effectively with ADP and P_i for $I \sim X$. A similar lag in the intensity-rate curve would be expected on the basis of postulate I. At low light intensity, the rate of formation of $I \sim X$ is low and thus would be directed preferentially toward ion transport.

If postulate II is correct, the "acid-bath phosphorylation" described by Jagendorf[24] can be attributed to the reversal of the mechanism for formation of the proton gradient; thus, ATP will be synthesized. This reaction is one of the primary experiments that supports the "chemiosmotic" hypothesis of phosphorylation in chloroplasts.

The results in chromatophores can be explained by assuming that the affinity of the proton or cation pump (reaction 1) for $I \sim X$ is not as great as in chloroplasts. Thus, ATP formation is the preferred reaction, and no time or intensity lag can be observed. To our knowledge, there are presently no experiments reported that distinguish between the two mechanisms if postulates I and II are correct.

Alternatively, it is possible that the intensity lag observed in chloroplasts is not related to ion transport. In light of the observation that an uncoupling

agent induced a lag in chromatophores (Fig. 4), we could argue that the lag in chloroplasts may be due to the presence of an endogenous uncoupler (such as a fatty acid) or other energy-dissipating reactions which compete favorably for $I \sim X$.

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